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TonB Interacts with Nonreceptor Proteins in the Outer Membrane of *Escherichia coli*

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The *Escherichia coli* TonB protein serves to couple the cytoplasmic membrane proton motive force to active transport of iron-siderophore complexes and vitamin B_{12} across the outer membrane. Consistent with this role, TonB has been demonstrated to participate in strong interactions with both the cytoplasmic and outer membranes. The cytoplasmic membrane determinants for that interaction have been previously characterized in some detail. Here we begin to examine the nature of TonB interactions with the outer membrane. Although the presence of the siderophore enterochelin (also known as enterobactin) greatly enhanced detectable cross-linking between TonB and the outer membrane receptor, FepA, the absence of enterochelin did not prevent the localization of TonB to the outer membrane. Furthermore, the absence of FepA or indeed of all the iron-responsive outer membrane receptors did not alter this association of TonB with the outer membrane. This suggested that TonB interactions with the outer membrane were not limited to the TonB-dependent outer membrane receptors. Hydrolysis of the murein layer with lysozyme did not alter the distribution of TonB, suggesting that peptidoglycan was not responsible for the outer membrane association of TonB. Conversely, the interaction of TonB with the outer membrane was disrupted by the addition of 4 M NaCl, suggesting that these interactions were proteinaceous. Subsequently, two additional contacts of TonB with the outer membrane proteins Lpp and, putatively, OmpA were identified by in vivo cross-linking. These contacts corresponded to the 43-kDa and part of the 77-kDa TonB-specific complexes described previously. Surprisingly, mutations in these proteins individually did not appear to affect TonB phenotypes. These results suggest that there may be multiple redundant sites where TonB can interact with the outer membrane prior to transducing energy to the outer membrane receptors.

The cell envelope of *Escherichia coli* and other gram-negative bacteria consists of two concentric membranes separated by an aqueous compartment through which nutrients must be transported. *E. coli* scavenges Fe(III)-bearing siderophores and vitamin B_{12} from the environment through a set of ligand-specific, high-affinity transport proteins displayed at the cell surface (outer membrane receptors). The subsequent release of the ligands from the outer membrane receptors collected in this manner into the aqueous periplasmic space is an active process dependent upon energy derived from the cytoplasmic membrane. The spatial separation of transport events from their energy source necessitates a mechanism for energy transfer. This need is met by TonB, a cytoplasmic membrane protein that spans the periplasmic space to transduce cytoplasmic membrane-derived energy to the outer membrane receptors (reviewed in references 4, 18, 19, 28, and 31).

TonB appears to shuttle between the cytoplasmic and outer membranes during the course of energy transduction (26). When the *E. coli* cell envelope is fractionated on sucrose density gradients, TonB is distributed approximately 60% and 40% between cytoplasmic and outer membranes, respectively. In these studies it was evident that the amino-terminal transmembrane domain of TonB (which also functions as an uncleaved signal sequence [33]) as well as cytoplasmic membrane proteins ExbB and ExbD (and TolQ/R) are required for TonB localization in the cytoplasmic membrane. The amino-terminal transmembrane domain is also required for export of TonB from the cytoplasm to the cytoplasmic membrane (37), cross-linking to ExbB in vivo (17, 23), and a conformational response of TonB to the proton motive force (22).

It had been previously shown that the carboxy terminus of TonB is required for association with the outer membrane (26). The outer membrane determinants involved in that association remain undefined. Thus, the studies in this paper were initiated to identify the outer membrane components that facilitate this interaction. We demonstrate here that, while cross-linking of TonB with the outer membrane receptor FepA is enhanced by the presence of its ligand, enterochelin, the presence or absence of enterochelin does not influence TonB distribution between the two membranes. Indeed, the absence of virtually all TonB-dependent outer membrane receptors failed to prevent localization of TonB to the outer membrane. The identification of two additional contacts of TonB at the outer membrane, the peptidoglycan-associated proteins Lpp and OmpA, suggests that the association of TonB with the outer membrane may be mediated through a set of proteins that provide docking sites prior to or following the energy transduction event.

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype or characteristic	Reference or source
GM1	*ara* Δ(*pro-lac*) *thi* F′ *pro lac*	40
W3110	F⁻ IN(*rrnD-rrnE*)1	14
KP1092	GM1 *fepA*::*kan*	36
KP1265	H455 *zhf-50*::Tn*10*	This study
KP1274	GM1 *aroB zhf-50*::Tn*10*	This study
KP1275	GM1 *fepA*::*kan aroB zhf-50*::Tn*10*	This study
KP1344	GM1 *tonB*::*blaM*	22
KP1357	GM1 Δ*lpp-254 zdh-925*::Tn*10*	This study
KP1359	JE5505 *zdh-925*::Tn*10*	This study
KP1364	JC7623 Δ*pal*::*cat*	This study
KP1369	KP1344 Δ*lpp-254 zdh-925*::Tn*10*	This study
KP1386	GM1 Δ*pal*::*cat*	This study
KP1387	KP1357 Δ*pal*::*cat*	This study
KP1390	KP1357 *tonB*::*blaM*	This study
KP1391	KP1390 Δ*pal*::*cat*	This study
KP1426	JF699 *zcc-282*::Tn*10*	This study
KP1427	GM1 *ompA252 zcc-282*::Tn*10*	This study
KP1431	KP1357 *zdh-3122*::Tn*10kan*	This study
KP1432	KP1431 *ompA252 zcc-282*::Tn*10*	This study
H455	*aroB pro lac malT tsx thi*	9
C1093	*araD* Δ(*lac*) *aroB rpsL relA flbB deoC ptsF rbsR* Δ(*chlA, fiu*) *fhuE*::Mu d1X *fecA*::Tn*1732 fepA cir fhuA*	8
JC7623	F⁻ *thi1 proA2 argE3 thr-1 leuB6 ara14 lacY1 galK xyl-5 mtl-1 supE44 tsx-33 rpsL31 recB1 recC22 sbcB15 spcC201*	44
JF699	F⁻ *lacY29 proC24 tsx-63 purE41* λ⁻ *ompA252 his-53 rpsL97 xyl-14 metB65 cycA1 cycB2*? *ilv-277*	10
JE5505	Δ(*gpt-proA*)62, *lacY1 tsx-29 glnV44 galK2* λ⁻ Δ*lpp-254 pps-6 hisG4*(*Oc*) *xylA5 mtl-1 argE3*(*Oc*) *thi-1*	15
CAG18466	MG1655 *zcc-282*::Tn*10*	35
CAG18450	MG1655 *zhf-50*::Tn*10*	35
CAG12151	MG1655 *zdh-925*::Tn*10*	35
CAG18568	MG1655 *zdh-3122*::Tn*10kan*	35
pKP315	pBAD18 expressing TonB	22
pKP379	pET24a-$TonB_{101–239}$	This study
pKP398	pJC417 *pal*::*cat*	This study
pJC417	pT7-5 expressing *tolB pal orf2*	43
pLysS	pACYC184 expressing the T7 *lysS* gene	Novagen

MATERIALS AND METHODS

Construction of bacterial strains and plasmids and growth conditions. Plasmids and bacterial strains used are summarized in Table 1. Strain KP1359 was created by linking the *zdh-925*::Tn*10* from CAG12151 to the Δ*lpp* from JE5505 by generalized transduction using $P1_{vir}$ (27). Tetracycline-resistant transductants retaining the Δ*lpp* marker were identified by sensitivity to media containing 0.4% sodium dodecyl sulfate and 1 mM EDTA (12). The Δ*lpp* phenotype was approximately 30% linked to Tn*10*. KP1357 and KP1369 were generated by subsequent $P1_{vir}$-mediated transduction of Δ*lpp zdh-925*::Tn*10* from KP1359 into GM1 and KP1344 (W3110, Δ*tonB*::*blaM*), respectively, and the genotypes were confirmed as above.

Plasmid pKP379 was created by PCR-mediated amplification of a region of TonB encoding amino acids (aa) 101 to 239 from pKP315 using primers oKP248 (5′-GATCTAGAAAGCCGGTGAAAAAGG-3′) and oKP249 (5′-GGTCTA GACTATTACTGAATTTCGGTGG-3′), corresponding to bp 637 to 652 and 1056 to 1040 of the published *tonB* sequence (32), respectively. The *Xba*I sites incorporated by the primers were used to clone the fragment into pET24-a at the compatible *Nhe*I site.

Strain KP1265 was created by linking the *zhf-50*::Tn*10* mutation from CAG18450 to the *aroB* mutation in H455 by generalized transduction using $P1_{vir}$. Tetracycline-resistant transductants retaining the *aroB* mutation were identified by screening on chromazurol S (CAS) medium (34). The *aroB* mutation was approximately 2% linked to Tn*10*. The *aroB zhf-50*::Tn*10* from KP1265 was then transduced into GM1 by generalized transduction using $P1_{vir}$ to create KP1274. To create strains KP1386 and KP1387, a Δ*pal*::*cat* mutant was first generated in a plasmid-encoded *tolB pal orf2* operon on pJC417 such that the *cat* open reading frame precisely replaced *pal*, presumably leaving *orf2* expression intact. Extra-long inverse PCR was used to amplify all of pJC417 except codons 2 to 172 of the *pal* gene. PCR was performed using primers oKP372 and oKP373 (5′ CATTT CAATGATTCCTTTACTATTC 3′ and 5′TAAGAGAATTGCATGAGCAGT AAC 3′) corresponding to bp 1613 to 1589 and 2130 to 2153 of the published *tol* operon, respectively (41). Codons 2 to 219 of the *cat* gene were amplified from pLysS using oKP370 (5′GAGAAGAAGATCACCGGCTATACC3′) and oKP371 (5′TGCACCTCCCTGCCATTCATCG3′) corresponding to bp 244 to 267 and 4448 to 4427 of pLysS, respectively. Underlined bases in the primers represent silent changes introduced to improve the percent GC balance between primers. Both *cat* gene primers were phosphorylated at the 5′ end. Plasmid pKP398 was demonstrated to encode TolB, BlaM, Orf2, and Cat but not Pal by in vitro transcription and translation using an *E. coli* S30 extract (Promega). Immunoblot analyses were performed to confirm these results using anti-Pal and anti-TolB antibodies (generous gifts of Jean-Claude Lazzaroni) and anti-BlaM antisera. To create strain KP1364, plasmid pKP398 carrying the Δ*pal*::*cat* mutation was linearized with *Hin*dIII and recombined into the chromosome of JC7623. Recombinants were selected on chloramphenicol and screened for sensitivity to ampicillin at 30 μg ml^{-1}. The resultant strain was confirmed to lack Pal, but retain TolB, by immunoblot analyses using anti-Pal and anti-TolB antibodies (data not shown). The Δ*pal*::*cat* mutation was then transduced by generalized transduction into GM1 and KP1357 using $P1_{vir}$, resulting in KP1386 and KP1387, respectively. KP1390 resulted from generalized transduction using $P1_{vir}$ of the *tonB*::*blaM* mutation in KP1344 into KP1357. KP1391 was made by generalized transduction using $P1_{vir}$ of the Δ*pal*::*cat* mutation from KP1364 into KP1390.

Strain KP1426 was created by linking the *zcc-282*::Tn*10* from CAG18466 to the *ompA* marker from JF699 by generalized transduction using $P1_{vir}$. Tetracycline-resistant transductants retaining the *ompA* marker were identified by resistance to bacteriophage K3 (38). KP1427 was then generated by generalized transduction using $P1_{vir}$ of *ompA*-deficient *zcc-282*::Tn*10* from KP1426 into GM1 with selection and screening as described above. The absence of OmpA was confirmed by its absence from the total protein profiles of KP1426 and KP1427 compared to that of the GM1 parental strain (data not shown).

Tryptone plates, T-top agar, and Luria-Bertani (LB) broth and agar were made as described previously (27). Liquid cultures were grown with aeration at 37°C in LB medium or in M9 minimal salts medium supplemented with 0.4% glucose, 40 μg of tryptophan/ml, 0.2% Casamino Acids, 0.4 μg of thiamine/ml, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, and 1.8 μM $FeCl_3 \cdot 6H_2O$. Antibiotics were used at the following concentrations unless otherwise indicated: ampicillin, 100 μg ml^{-1}; kanamycin, 20 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; chloramphenicol, 34 μg ml^{-1}.

Chemicals and reagents. Enzymes, primers, and sodium dodecyl sulfate were purchased from Gibco BRL (Grand Island, N.Y.). DNA purification kits were purchased from Qiagen (Valencia, Calif.). Anti-TonB monoclonal antibodies 4F1 and 4H4 have been described previously (20). Media components were purchased from Difco Laboratories (Detroit, Mich.). Enhanced chemiluminescence (ECL) immunoblotting kits were purchased from NEN Life Sciences (Boston, Mass.). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G was purchased from Caltag Laboratories (Burlingame, Calif.). Polyvinylidene fluoride membranes (Immobilon-P) were purchased from Millipore Corp. (Bedford, Mass.). Acrylamide and trichloroacetic acid were purchased from Fisher Biotech (Park Lawn, N.J.), and bis-acrylamide was from Bio-Rad Laboratories (Richmond, Calif.). The protease inhibitor cocktail (Complete) was purchased from Boehringer Mannheim (Indianapolis, Ind.). The 16% paraformaldehyde (electron microscopy grade) was purchased from Electron Microscopy Sciences (Ft. Washington, Pa.). Glass fiber filters were purchased from Whatman (Maidstone, England), and liquid scintillation fluid was purchased from Amersham Pharmacia Biotech (Piscataway, N.J.). All other reagents were purchased from Sigma (St. Louis, Mo.).

Sucrose density gradients. TonB localization with cytoplasmic or outer membranes was determined by sucrose density gradient fractionation as described previously (26). To test the effect of high salt and chaotropic agents on TonB localization, lysates produced by French press were adjusted to 4 M KCl, 9 M guanidine-HCl (GnHCl), or 4 M urea immediately prior to application to the sucrose gradients. In the case of GnHCl and urea, it was not possible to monitor NADH oxidase as a marker for the cytoplasmic membrane. Instead, the fractions containing the cytoplasmic membrane were identified in immunoblots by the presence of the magnesium transporter CorA by using anti-CorA antibody, a generous gift of Michael Maguire. The absence of iron-responsive outer membrane proteins (IROMPs) in strain C1093 was confirmed in the immunoblot of the outer membranes, which was stained for total protein (data not shown).

In vivo formaldehyde cross-linking analysis. Bacteria in logarithmic growth phase were cross-linked in vivo with 1% monomeric formaldehyde as described previously (13). To test the effects of ferric-enterochelin on the ability of TonB to cross-link to FepA, cells were harvested at mid-log phase, pelleted, and suspended in 1 ml of filter-sterilized (0.2-μm-pore-size filter) conditioned M9 minimal medium from saturated Δ*tonB* cultures (which hypersecrete enterochelin) or from Δ*tonB aroB* cultures (which cannot synthesize enterochelin or any relevant enterochelin precursors). Cells were incubated with aeration in these media for 10 min at 37°C and then processed for cross-linking as described previously (13), but using reagent-grade formaldehyde (J.T. Baker, Philipsburg, N.J.) was used.

Purification of $TonB_{101-239}$. Strain BL21(λDE3) carrying pKP379 was grown in LB medium to late log phase and then induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside for 3 h. The bacteria were pelleted at 4°C and lysed in a French press at 20,000 lb/in^2. All subsequent operations were carried out at 4°C. The lysate was centrifuged at 40,000 × *g* for 30 min, and the supernatant was applied to an HQ column (10 by 100 mm; Applied Biosystems) which was equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 10 mM NaCl, using a BioCAD Sprint apparatus (Applied Biosystems). Fractions eluting between 100 and 200 mM NaCl were pooled, concentrated with a YM-10 membrane (Millipore), and dialyzed against 20 mM Tris-HCl buffer, pH 9.0, containing 10 mM NaCl. The sample was then loaded onto a MonoS HR 5/5 column using a fast-performance liquid chromatography system (Amersham Pharmacia Biotech). Pure TonB protein eluted with about 400 mM NaCl and was concentrated with a Centriplus −10 (Amicon), and the buffer was exchanged to 20 mM Tris-HCl, pH 7.6. The final concentration of the $TonB_{101-239}$ protein was 5 mg/ml (data not shown).

In vitro formaldehyde cross-linking analysis. To determine the localization of various cross-linked TonB complexes, bacterial cultures were grown in minimal medium in the absence of iron and concentrated in the presence of a protease inhibitor cocktail (Complete). They were then lysed by French press and fractionated on sucrose density gradients. Cytoplasmic membrane fractions (as identified by peak NADH oxidase activity) were then cross-linked as described previously (36). Outer membrane fractions (as identified by peak absorbance at A_{280} in fractions containing a density greater or equal to 1.2 gm/cm^3) were diluted with an equal volume of 10 mM HEPES buffer, pH 7.8. Reagent-grade formaldehyde (37%) was added to a final concentration of 1%, and samples were incubated at room temperature for 15 min and then precipitated with 5 volumes of acetone (−20°C). After 10 min at −20°C, the samples were pelleted at 4°C, air dried, and suspended in Laemmli sample buffer.

To analyze the ability of purified $TonB_{101-239}$ to cross-link to Lpp in vitro, KP1344 (Δ*tonB*::*blaM*) and KP1369 (Δ*tonB*::*blaM* Δ*lpp*) were fractionated on sucrose density gradients as described previously, and the respective outer membrane fractions were pooled separately. Outer membranes from 25 ml of cells harvested at an A_{550} of 0.5 (as determined on a Spectronic 20 spectrophotometer; path length = 1.5 cm) were mixed with 5 μg of purified $TonB_{101-239}$ in 100 mM sodium phosphate buffer, pH. 7.8, and incubated on ice for 15 min. The samples were then diluted sixfold with sodium phosphate buffer, and formaldehyde was added to a final concentration of 1%. After vortexing, the samples were incubated for 30 min on ice. The membranes were recovered by centrifugation at 322,000 × *g*. The pellets were then analyzed by immunoblotting.

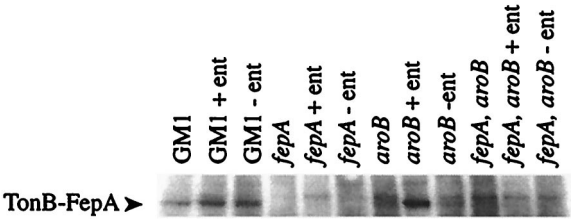

FIG. 1. Enterochelin enhances formation of the TonB-FepA complex in vivo. Cross-linking profiles of strains cross-linked without addition, or in the presence of added sterile cell supernatants containing enterochelin (+ent), or not containing enterochelin (−ent). Note that GM1 and its *fepA* derivative also synthesize enterochelin endogenously. All strains analyzed were isogenic derivatives of GM1. A region of an immunoblot developed with the anti-TonB antibody 4F1 is shown. The arrow indicates the position of the TonB-FepA complex at 195 kDa.

Immunoblotting analysis of proteins. Immunoblotting analyses were then performed as previously described with the monoclonal anti-TonB antibodies 4F1 and 4H4 (20).

Activity assays. Relative TonB activity was determined as a measure of [^{55}Fe] ferrichrome transport, as modified previously (21). Briefly, 1.65 ml of mid-log cultures were grown in minimal medium without added iron, pelleted at room temperature, and resuspended in 13 ml of M9 minimal medium containing 0.1 mM nitrilotriacetic acid. Cells were equilibrated at 37°C with shaking for 5 min, and transport was initiated by adding 300 pmol of [^{55}Fe]ferrichrome (prepared by preincubation of deferrated ferrichrome and [^{55}Fe]Cl_3 at a molar ratio of 6.6:1 in 10 mM HCl for 15 min at 37°C). To assay transport, triplicate 0.4-ml samples were taken at the times indicated and cells were collected on Whatman GF/C filters and then washed three times in 5 ml of 0.1 mM LiCl. Filters were air dried, equilibrated in liquid scintillation fluid overnight, and counted for 1 min. Bacteriophage and colicin sensitivity assays were performed as described previously (22).

RESULTS

The presence of enterochelin enhances TonB interaction with the outer membrane receptor FepA. It had previously been demonstrated that TonB interactions with the outer membrane receptor FhuA are enhanced in the presence of one of its transport ligands, ferri-ferricrocin, both in vivo and in vitro (29). Interactions between FepA and TonB are enhanced by the presence of ligand in vitro (30). To determine whether this was also the case for enterochelin in vivo, the ability of TonB to cross-link in vivo in *aroB* cells, in the presence and absence of exogenously provided enterochelin, was examined (Fig. 1). *AroB* strains can synthesize neither enterochelin nor any precursors specific to the enterochelin biosynthetic pathway. As seen previously (36), the TonB-FepA cross-linked complex was entirely absent from *fepA* strains. As predicted, the level of TonB-FepA complex in the absence of enterochelin was greatly diminished. Consistent with previous results, the extent of TonB-FepA cross-linking detectable in vivo was significantly increased in the presence of enterochelin, whether provided endogenously or exogenously (Fig. 1).

Neither ligand nor outer membrane receptors are required

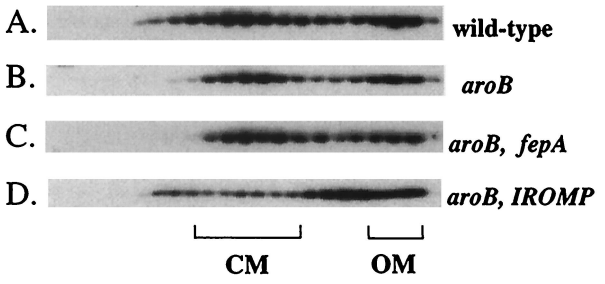

FIG. 2. The absence of enterochelin and IROMPs does not prevent TonB association with the outer membrane. Bacteria were lysed by French press, and the intact lysates were resolved on sucrose density gradients. An immunoblot developed with the anti-TonB antibody 4F1 is shown. (A) GM1; (B) KP1274 (GM1, *aroB*); (C) KP1092 (GM1, *fepA*::*kan*); (D) C1093 (*aroB*, lacking all IROMPs). OM indicates the position of the outer membrane fractions. CM indicates the position of the cytoplasmic membrane fractions. The IROMPs were absent from the outer membrane fractions (data not shown).

for TonB fractionation with the outer membrane. At steady state, approximately 40% of TonB in French press lysates fractionates with the outer membrane in sucrose density gradients (26). If the sole contact of TonB with the outer membrane occurred through FepA or additional TonB-dependent outer membrane receptors, TonB association with the outer membrane should be preventable by growth in the absence of a transportable ligand, i.e., in an *aroB* strain (45). Surprisingly, the absence of enterochelin did not decrease TonB association with the outer membrane. Nor did the absence of FepA, nor indeed all of the outer membrane receptors for siderophores (IROMPs) (Fig. 2). In fact, the absence of all the IROMPs appeared to increase the proportion of TonB at the outer membrane, with TonB also spreading into lower-density fractions intermediate to the cytoplasmic membrane and outer membrane. Together, these results suggested that there were other sites at the outer membrane, in addition to the TonB-dependent outer membrane receptors, where TonB could interact. The treatment of a French press-generated lysate of GM1 with lysozyme (1.8 mg per 100 ml of cells at an A_{550} of 0.5) prior to fractionation on sucrose gradients did not remove TonB from the outer membrane (data not shown). In addition, TonB fractionated similarly between cytoplasmic and outer membranes in bacteria that had been converted to spheroplasts by lysozyme treatment and then either sonicated or osmotically lysed. TonB also appeared in the M-band under those conditions, consistent with the presence of both outer membranes and cytoplasmic membranes in that fraction (data not shown).

TonB can be removed from the outer membrane but not the cytoplasmic membrane by high salt and chaotropic agents. In the absence of the TonB-dependent outer membrane receptors, TonB could be interacting at the outer membrane with protein, lipid, or both. To characterize the biochemical nature of TonB interactions at the outer membrane, French press lysates of GM1 (*tonB*$^+$) were treated with either 4 M KCl, 9 M GnHCl, or 4 M urea prior to loading on sucrose density gradients. The fractionation profile of untreated GM1 confirmed our previous finding of TonB in both the cytoplasmic and outer membranes (Fig. 3). Addition of 4 M KCl to the lysate significantly depleted the proportion of TonB in the outer membrane and populated the soluble protein region of the gradient with TonB and a degradation product characteristic of outer membrane-localized TonB. Conversely, the cytoplasmic membrane TonB pool was more refractory to this treatment, remaining membrane associated. Similar results were seen for GnHCl and urea (data not shown). The simplest interpretation of the results is that TonB interactions at the outer membrane involve protein-protein contacts, while those in the cytoplasmic membrane might include transmembrane domain interactions with lipids.

The 43- and 77-kDa TonB-specific complexes are found in the outer membrane. TonB has been shown to form cross-links in vivo with other, as yet unidentified, proteins (36). To deter-

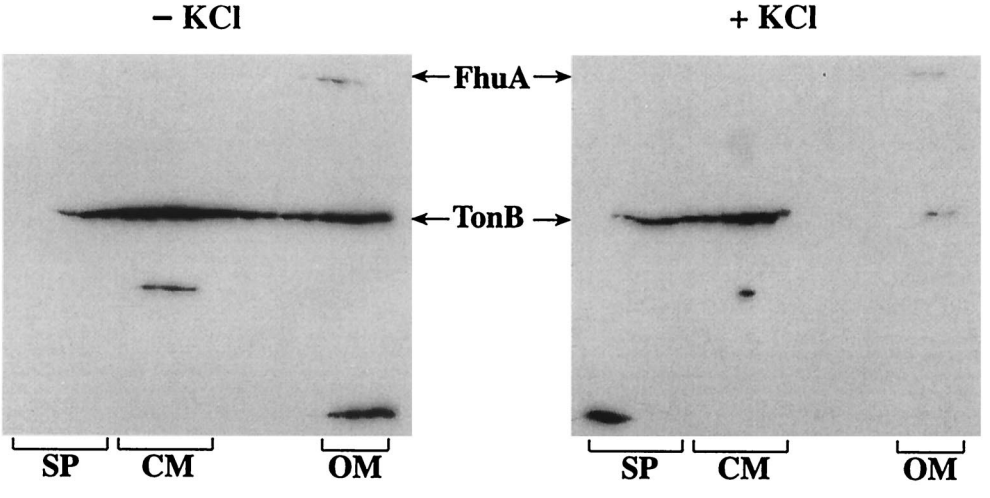

FIG. 3. High salt removes TonB from association with the outer membrane but not the cytoplasmic membrane. Bacteria were lysed by French press and half of the lysate was treated with 4 M KCl while the other half was untreated. The lysates were then resolved on sucrose density gradients. Immunoblots of the gradient fractions developed with the anti-TonB antibody and anti-FhuA antibody are shown. OM indicates the position of the outer membrane fractions. CM indicates the position of the cytoplasmic membrane fractions. SP indicates the fractions containing soluble proteins. The positions of outer membrane protein FhuA and of TonB are indicated by arrows.

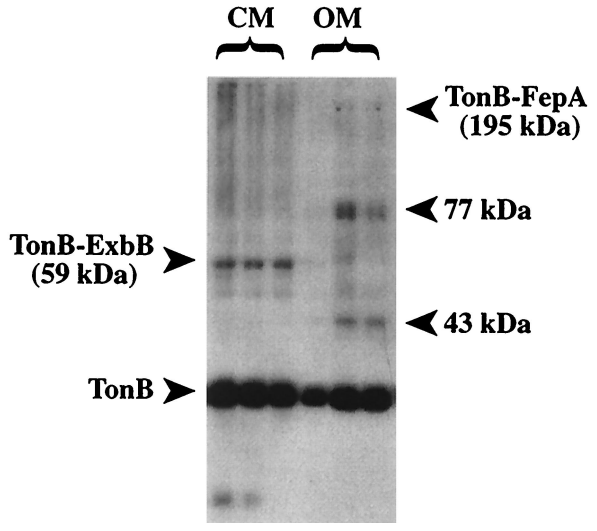

FIG. 4. Localization of TonB complexes to the outer membrane and the cytoplasmic membrane. Individual cytoplasm membrane and outer membrane fractions were treated with formaldehyde. Immunoblots of the cross-linking profiles were developed with anti-TonB antibody 4H4. The position of TonB monomer and the TonB-specific complexes are indicated. OM indicates the position of the outer membrane fractions. CM indicates the position of the cytoplasmic membrane fractions.

mine where these complexes occur, French press lysates were fractionated on sucrose density gradients and the cytoplasmic membrane and outer membrane fractions were subsequently cross-linked in vitro using 1% formaldehyde (Fig. 4). Of the four complexes originally observed by us in vivo (36), the TonB-ExbB complex was found in the cytoplasmic membrane as expected, while the 43-kDa, 77-kDa, and TonB-FepA complexes were found in the outer membrane. The 43- and 77-kDa complexes thus reflected at least a subset of the additional sites through which TonB could be interacting at the outer membrane.

The 43-kDa complex requires Lpp for its formation. To identify the components of the 43- and 77-kDa complexes, we began by assuming that the apparent molecular mass of TonB (36 kDa) could be subtracted from each complex to predict the approximate apparent molecular mass for the additional protein component. In the case of the 43-kDa complex, that approximation suggested a 7-kDa protein, for which the best candidate was the lipoprotein Lpp. To determine if Lpp played a role in formation of the 43-kDa complex, an isogenic Δ*lpp* strain was cross-linked in vivo (Fig. 5), with the result that the 43-kDa complex was indeed absent.

Mutations in *lpp* do not appear to affect TonB-dependent energy transduction. To determine the effects of an *lpp* mutation, the sensitivity of various strains to colicins and ϕ80, the ability of the strains to transport ferrichrome, the fractionation of TonB, and the half-lives of TonB in the mutant strains were measured (Fig. 6 and data not shown). Surprisingly, the *lpp* mutation had no significant effect in any of the assays. It seemed likely that Pal, peptidoglycan-associated lipoprotein, which plays an important role in the analogous Tol system (7), might substitute for Lpp. To test that possibility, a *pal* strain and a *pal-lpp* strain were cross-linked. The *pal* mutation had little or no effect by itself or in combination with the *lpp* mutation in the cross-linking where a Pal-TonB complex was not evident (Fig. 5) or in other phenotypic assays (data not shown), beyond a slight increase in the rate of ferrichrome uptake (Fig. 6).

Lpp forms a complex with the carboxy terminus of TonB in vitro. To test whether TonB could interact with Lpp in vitro, the purified carboxy-terminal domain of TonB (aa 101 to 239) was mixed with the outer membrane fractions of both wild-type and *lpp* bacteria and then cross-linked with monomeric formaldehyde (Fig. 7). A TonB-specific complex was formed at 27.5 kDa in the *lpp*$^+$ outer membranes that was absent from the *lpp* outer membranes. The smaller Lpp-specific band was most likely a degradation product, since a similar band was apparent when the experiments shown in Fig. 4 were performed in the absence of protease inhibitors (data not shown). The migration of this complex was consistent with a composition of one Lpp molecule and one molecule of $TonB_{101-239}$. The other prominent bands in the cross-linking profile appeared to arise from interactions of the TonB carboxy terminus with itself, since they occurred in similar experiments performed with the cytoplasmic membrane (data not shown) and in the lane where only the carboxy terminus of TonB was present (Fig. 7). In particular, a dimer and possible trimer of the carboxy-terminal domain appeared to be present.

Part of the 77-kDa cluster requires OmpA, another peptidoglycan-associated protein, for its formation. In the case of the 77-kDa complex, which appears most often as a cluster of cross-linked complexes with a prominent band at 77 kDa (36), subtraction of the apparent molecular mass of TonB left a protein with a predicted molecular mass of approximately 41

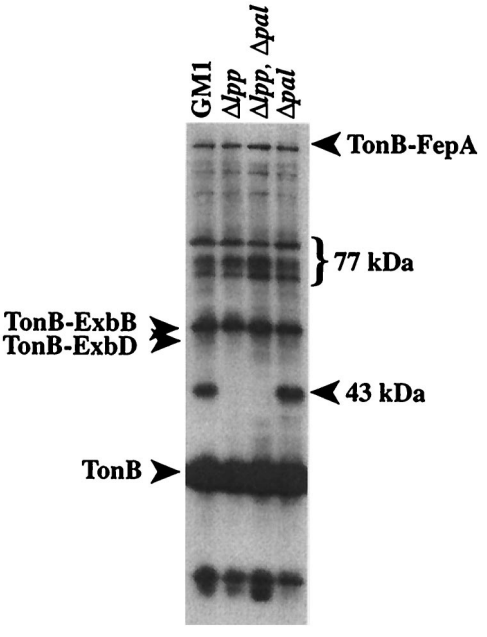

FIG. 5. The 43-kDa TonB-specific complex is absent from the cross-linking profile of a Δ*lpp* strain. Immunoblot with the anti-TonB antibody 4H4 is shown. TonB monomer and TonB-specific cross-linked complexes are indicated by arrows. All strains used are isogenic derivatives of GM1.

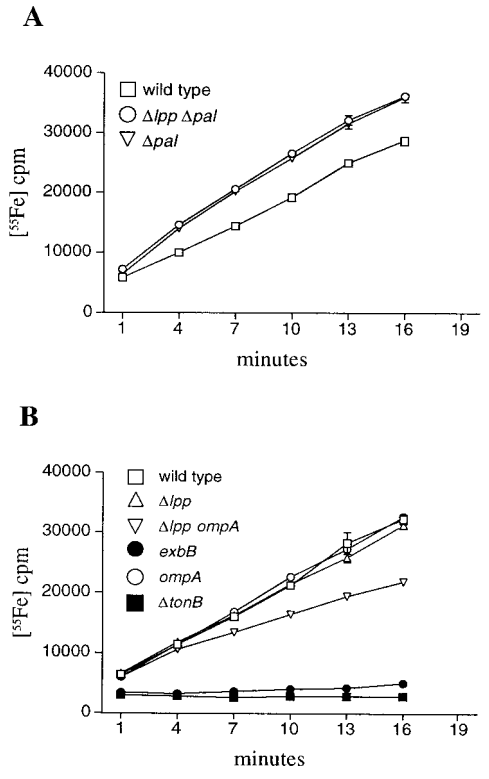

FIG. 6. TonB activity in mutant backgrounds. [^{55}Fe]ferrichrome transport into whole cells was measured. Triplicate samples were taken at the indicated times, and the standard deviations are represented by error bars, which are only shown where the values exceed the resolution of the symbols. Data presented are from one experiment; however, transport rate relationships between wild type (wt) and mutants were preserved in duplicate experiments. Rates of transport were calculated over 16-min intervals from the slopes derived by linear regression as follows: (A) wt, 1,554 ± 24; Δ*pal*, 1,958 ± 52; Δ*lpp* Δ*pal*, 1,936 ± 51. (B) wt, 1,751 ±62; Δ*lpp*, 1,614 ± 32; *ompA*, 1,771 ± 33; Δ*lpp ompA*, 1,036 ± 33; *exbB*, 103 ± 15; Δ*tonB*, −6 ± 7;

kDa. It has been previously suggested that the 77-kDa complex does not contain a TonB dimer (24). Since the apparent molecular mass of any cross-linked complex can be affected by the position of the cross-links, outer membrane proteins in the molecular mass range of 30 to 50 kDa were considered. Among the candidates were OmpA, OmpC, and OmpF. While the absence of OmpF and OmpC did not affect the cross-linking profile (data not shown), the absence of OmpA resulted in the loss of the two lower bands in the 77-kDa cluster, leaving the most prominent band in place and still unidentified (Fig. 8). Interestingly, the intensity of the TonB-FepA cross-linked band appeared to increase significantly in an Δ*lpp ompA* double mutant, although it remained a minor component of the cross-linking profile. Ferrichrome transport assays of the OmpA mutant and its isogenic parent revealed no effect of the OmpA mutation. However, the double Δ*lpp ompA* mutant showed ≈40% reduced ability to transport ferrichrome (Fig. 6). This loss of transport ability was most likely not due to shedding of outer membrane receptors by blebbing of the outer membrane, since the Δ*lpp pal* strain, which transported ferrichrome even more efficiently than the parental strain GM1, blebs an equal amount of outer membrane and FhuA compared to the Δ*lpp ompA* strain (data not shown). Consistent with, and probably due to, the equal outer membrane blebbing of the two strains, the sensitivities to TonB-independent bacteriophages T5, λ, and $P1_{vir}$ were similarly reduced in both double mutants (data not shown). Nonetheless, there was a slight diminution of FhuA expression in the Δ*lpp ompA* strain compared to the parent, and it was, thus, not possible to absolutely attribute the decrease in ferrichrome transport to a decrease in TonB function (data not shown).

DISCUSSION

The TonB protein serves to transduce energy from cytoplasmic membranes to outer membranes of gram-negative bacteria to allow active transport of iron siderophores and vitamin B_{12} through transporters located in the outer membrane. Results from in vivo cross-linking experiments previously demonstrated that TonB interacts closely with at least five proteins during this process: the outer membrane receptor FepA, cytoplasmic membrane proteins ExbB and ExbD, and at least two

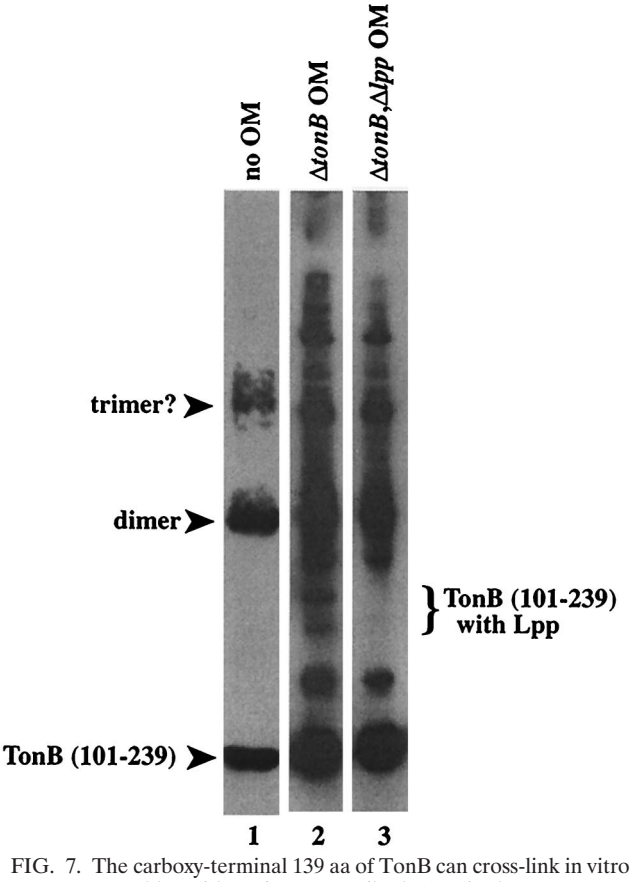

FIG. 7. The carboxy-terminal 139 aa of TonB can cross-link in vitro to Lpp. Immunoblot with anti-TonB antibody 4F1 is shown. Lane 1, purified $TonB_{101–239}$ was cross-linked with formaldehyde; lane 2, purified $TonB_{101–239}$ was added to purified outer membranes from KP1344 (Δ*tonB*::*blaM*) and cross-linked; lane 3, purified $TonB_{101–239}$ was added to purified outer membranes from KP1369 (Δ*tonB*::*blaM* Δ*lpp*) and cross-linked. The position of $TonB_{101–239}$ and a possible dimer and trimer are indicated by arrows. The pair of bands corresponding to the complex of $TonB_{101–239}$ with Lpp and a proteolytic product is indicated by a bracket.

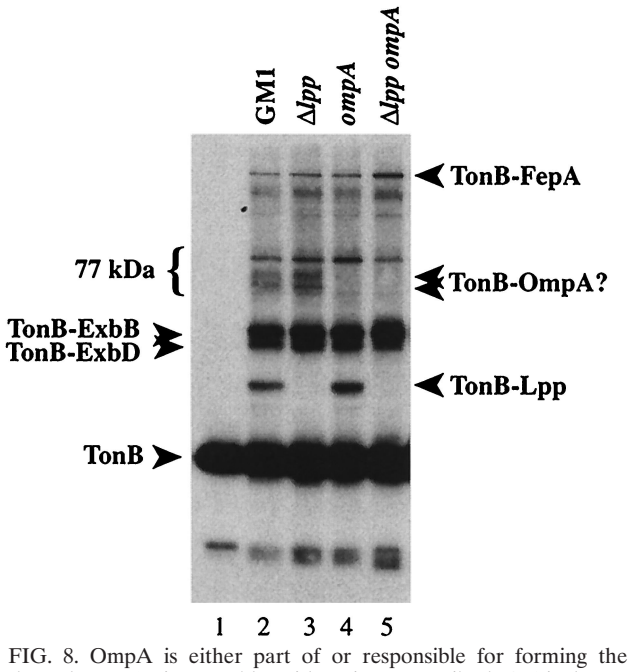

FIG. 8. OmpA is either part of or responsible for forming the 77-kDa cluster. An immunoblot with anti-TonB antibody 4F1 is shown. Lane 1, uncrosslinked GM1; lanes 2 to 5, in vivo cross-linking profiles of the indicated strains. The position of TonB monomer and TonB-specific cross-linked complexes are indicated by arrows.

unknown proteins which combine with TonB to form a 43-kDa complex and a cluster of complexes at 77 kDa, respectively (13, 36).

Previously, a significant amount of TonB-FepA cross-linked complex was observed in vivo in an *entF* bacterial strain that lacked the ability to synthesize enterochelin and was negative on CAS plates used to detect siderophore secretion (36). Based on that evidence, we concluded that ligand was not required for TonB to cross-link to FepA. However, in hindsight, since EntF acts late in the enterochelin biosynthetic pathway as part of the enterochelin synthase (11, 46), it seemed possible that intermediates in the enterochelin biosynthetic pathway could serve to signal ligand occupancy yet not be reactive on CAS plates. Consistent with that idea, it has also been more recently shown that the degree of TonB interaction with FhuA in vivo and in vitro (29), and with FepA in vitro (30), is significantly enhanced in the presence of ligand. To revisit the question of ligand effects on TonB-FepA interactions in vivo, *aroB* strains, which cannot synthesize any relevant biosynthetic intermediates of the enterochelin pathway, were cross-linked. Small amounts of TonB-FepA complexes were observable under these circumstances; however, the amount of complex increased significantly upon the addition of enterochelin, thus confirming previous results demonstrating that TonB interacts more detectably with liganded receptors. Indeed, recent experiments from this lab have shown that TonB undergoes a conformational change at the outer membrane in the presence of ligand, and this change does not occur in its absence (22).

The data in this study show that the presence of ligand was not the sole determinant of the ability of TonB to fractionate with the outer membrane. Even the absence of virtually all of the known TonB-dependent outer membrane receptors failed to prevent association with the outer membrane. It seemed unlikely that the sole remaining outer membrane receptor in strain C1093—BtuB which transports vitamin B_{12}—could have been responsible for TonB association with the outer membrane in the strain depleted of IROMPs (8). First of all, there was no vitamin B_{12} in the growth medium. Secondly, at ≈200 copies per cell, the BtuB protein is probably the least abundant of the TonB-dependent receptors. Lysozyme treatment did not prevent TonB association with the outer membrane fraction, making it unlikely that peptidoglycan was the source of the associative interaction. Furthermore, the association of TonB with the outer membrane could be disrupted with high salt, which is most consistent with the idea of protein-protein interactions as the sole determinant of its localization there. In contrast, the interaction of TonB with the cytoplasmic membrane was relatively insensitive to the action of high salt or even the chaotropic agents GnHCl and urea, suggesting that the nature of the interaction was different from that of TonB with the outer membrane. Thus, it appeared that TonB was making contacts with nonreceptor proteins in the outer membrane.

Two additional contacts in the outer membrane were identified when the 43- and 77-kDa TonB-dependent complexes localized to purified outer membranes. In order to begin to identify the unknown proteins in those complexes, a number of candidate mutants were cross-linked in vivo, based on an initial assumption that the molecular masses of two proteins in the complex might be quantitatively additive. The 43-kDa complex was absent from an *lpp*-deficient strain, suggesting that Lpp is either in the complex or responsible for forming it. Given the characteristic and unusually small molecular mass of Lpp (7 kDa), it seems highly likely that the 43-kDa complex contains Lpp.

The absence of Lpp had no detectable effect on the TonB phenotype by any of the usual TonB-specific assays. This result was unexpected, since the complexes of TonB with FepA, ExbB, and ExbD all represent proteins involved in TonB function. Since there is cross talk between ExbB and ExbD of the TonB system and TolQ and TolR of the Tol system (3), it seemed reasonable to ask whether Pal, a peptidoglycan-associated lipoprotein from the analogous Tol system (reviewed in reference 25), was substituting for Lpp. This was not the case, since the phenotype of the *lpp pal* double mutant was wild type with respect to TonB function. The possibility that the wild-type levels of sensitivity or transport observed were due to leakage through a damaged outer membrane—a characteristic of *lpp* or *pal* strains (1)—was eliminated by demonstrating that a *tonB* version of the *lpp pal* double mutant, KP1391, was completely inactive in TonB-specific assays (data not shown). Nonetheless, the plethora of lipoproteins present in *E. coli* (estimated to be more than 80 [42]) could result in redundancy of function and our inability to detect a phenotype. A putative TonB-Lpp cross-linked complex is conserved over a range of gram-negative bacteria (20), suggesting that it is functionally significant. In addition, the fact that the purified carboxy-terminal 139 aa of TonB could be cross-linked to Lpp in outer membranes in vitro suggested that the interaction was not an opportunistic one and defined an initial region of interaction with Lpp. The ability of $TonB_{101-239}$ to productively interact

with outer membranes also suggested that the protein fragment retained at least some of its native structure. Interestingly, $TonB_{101-239}$ was able to cross-link in vitro into dimers, consistent with the recent crystal structure of part (aa 164 to 239) of the TonB carboxy terminus (6).

Several mutations were screened for the possibility that they might affect formation of the 77-kDa cluster. Two protein bands in the 77-kDa cluster depended on the presence of OmpA, apparently also a peptidoglycan-associated protein. This suggests that OmpA is either in the complexes (the molecular mass is appropriate) or else it is required to form them. There was no evidence for an effect of the *ompA* mutation on TonB activity, either singly or in combination with the Δ*lpp* mutation. TonB did not cross-link to OmpF, OmpC, or TolC (data not shown); however, that is not evidence for a lack of interaction, since cross-linking will fail to detect any interactions where the appropriate amino acids are not in correct proximity to one another. A Δ*lpp ompA* version of C1093 missing all the IROMPs was not constructed due to a lack of available antibiotic resistance markers and the relative fragility of the C1093 strain. However, neither the Δ*lpp ompA* nor Δ*lpp ompA* double mutation affected the ability of TonB to associate with the outer membrane, as evidenced by the ability of TonB in those mutant backgrounds to cross-link with FepA.

Why does TonB cross-link with two peptidoglycan-associated proteins, both of which are required for keeping the outer membrane attached to the cell (39)? If TonB were acting nonspecifically with Lpp and OmpA, by sheer mass action the abundance of those two proteins (7×10^5 [2, 16] and $\approx 10^5$ per cell, respectively) would be expected to skew cross-linking distribution in favor of the complexes containing those proteins. Instead, the level of TonB cross-linking with each of the five proteins to which it can cross-link, including Lpp and a protein tentatively identified as OmpA, seems somewhat proportionate, with none being significantly more abundant than the others in wild-type cells. This is consistent with the idea that TonB determines the level of complex formation, and TonB spends an approximately equal amount of time interacting with each protein. The cross-linking of TonB with OmpA and Lpp is also consistent with the observation that TonB can associate with the outer membrane in the almost complete absence of TonB-dependent outer membrane receptors and in the complete absence of any transport ligands. Perhaps Lpp and OmpA serve as two of several docking sites where TonB can await the signal that the outer membrane receptors have been visited by ligands. From a docking site at the outer membrane, TonB could then transduce energy to a newly liganded outer membrane receptor. These findings are certainly consistent with the findings in the analogous Tol system, where TolA, the TonB analogue, has been shown to interact with the outer membrane lipoprotein Pal (5).

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